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SHORT COMMUNICATION

ISOLATION OF GLUCOSAMINE 6-PHOSPHATE FROM THE CELL WALLS OF *MICROCOCCUS LYSODEIKTICUS*[1]

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Muramic acid 6-phosphate was first demonstrated by Liu and Gotschlich (1963, 1967) to be an essential component of bacterial cell walls, and was later shown to serve as an anchor of peptidoglycans to special structures, such as antigenic polysaccharides and teichoic acids.

In studies on the chemical structure of *Corynebacterium diphtheriae* cell walls, we obtained evidence that an arabinogalactan, a special structure of these cell walls, was linked to a glycan portion of peptidoglycan through muramic acid 6-phosphate, and so we attempted to prepare muramic acid 6-phosphate as a reference. We used the supernatant fluid of protoplasts of *Micrococcus lysodeikticus* as a starting material. The concentrated supernatant was hydrolyzed in 6 N HCl, at 100 C for 60 min, and the hydrolyzate was chromatographed on a Dowex-50 (H^+ form) column, by the method of Liu and Gotschlich (1967). However, in this way we could not separate muramic acid 6-phosphate from other contaminating ninhydrin-positive phosphate compounds. One of these contaminants yields glucosamine on treatment with alkaline phosphatase (Type III-S, from *E. coli*, Sigma, St Louis, Mo., USA). This and other preliminary findings suggesting that this contaminant was glucosamine 6-phosphate were reported previously (Kato, et al., 1972: Kato and Kotani, 1974). This paper reports the large scale preparation of this compound and its further identification.

Purified cell walls of *M. lysodeikticus* isolated by the conventional method (Iwata, et al., 1972) were used as starting material. One gram of cell walls was hydrolyzed in 100 ml of 6 N HCl at 100 C for 60 min, and the hydrolyzate was evaporated under reduced pressure. The residue was dissolved in 10 ml of distilled water, and insoluble materials were removed by filtration through a sintered glass filter. The filtrate was concentrated to 5 ml and applied on a Dowex-50×8 (H^+ form, 200–400 mesh) column (1×28 cm), and the column was eluted with deionized water. Fractions of 5 ml were collected and aliquots were tested for total hexosamines and phos-

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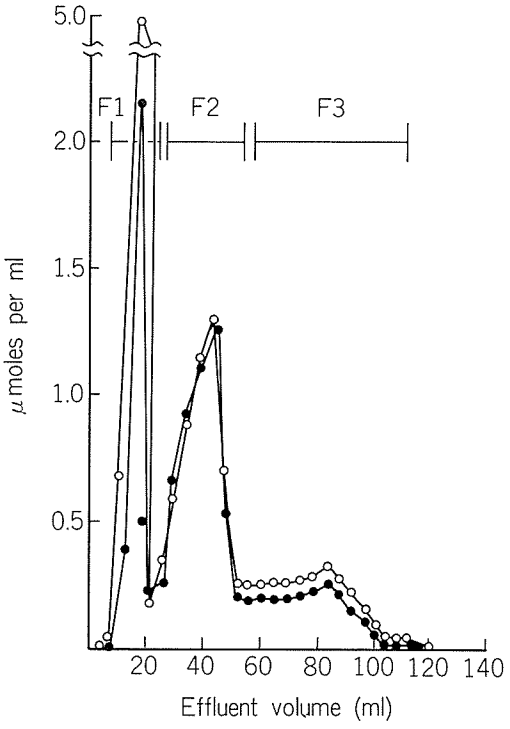

FIGURE 1. Chromatography of the *M. lysodeikticus* cell wall hydrolyzate on a Dowex-50×8 column. The column was eluted with deionized water. Column size: 1×28 cm. Total hexosamines (○——○), total phosphates (●——●).

phates. Total hexosamines, and inorganic and total phosphates were determined by the method of Ghuysen et al. (1966) and Lowry et al. (1954), respectively. As shown in Fig. 1, the eluate was divided into three portions (Fractions I, II and III) on the basis of the contents of hexosamines and phosphates. These portions were each concentrated to 2 ml, and then submitted to amino acid analysis with a Hitachi amino acid auto-analyzer (KLA-3B, Hitachi Ltd., Tokyo), before and after digestion with alkaline phosphatase. Muramic acid 6-phosphate (kindly supplied from Dr. T. Osawa, Division of Chemical Toxicology and Immunochemistry, Faculty of Pharmaceutical Sciences, University of Tokyo and Dr. K. Yamamoto, Department of Chemistry, Osaka University, Faculty of Science) and glucosamine 6-phosphate (Grade 1, Sigma) were used as authentic preparations. It was found that the major components of Fractions II and III were muramic acid 6-phosphate and glucosamine phosphate, respectively. Fraction I contained roughly equimolar amounts of muramic acid and glucosamine after treatment with phosphatase, but since very little material was recovered in this fraction, it was not analyzed further.

Fractions II and III (1.5 ml each) were rechromatographed on Dowex-50×8 (H^+ form) column (1×32 cm) (Fig. 2). The elution patterns in terms of total hexosamines and total phosphates showed that the major component of Fraction II was muramic acid phosphate and that Fraction III was composed almost entirely of glucosamine phosphate. The material eluted with 85–140 ml of effluent (GP fraction in Fig. 2) was concentrated and its amino acid composition was analyzed before and after incubation with alkaline phosphatase at 37 C for 1 hr. Before enzyme treatment it gave an acidic compound with a retention time of 22 min on the long column of the analyzer, coinciding with that of authentic glucosamine 6-phosphate. After phosphatase-treatment the specimen gave only a peak of glucosamine without any detectable material with a retention time of 22 min. The retention time of muramic acid 6-phosphate on this long column of the analyzer was 18 min. Buffer of pH 5.28, instead of pH 3.25 was used in amino acid analyzer to obtain clear separation of the peak of glucosamine from those of galactosamine and mannosamine. When the glucosamine phosphate was heated at 100 C in 1 M $HClO_4$ for 45 min a trace amount of inorganic phosphate was liberated, excluding the possibility that the material was the acid-labile compound glucosamine 1-phosphate. The position of the phosphate ester on the glucosamine residue was identified by measuring formation of glycolaldehyde phosphate on oxidation of the compound with sodium metaperiodate, using the method of Liu and Gotschlich (1967)

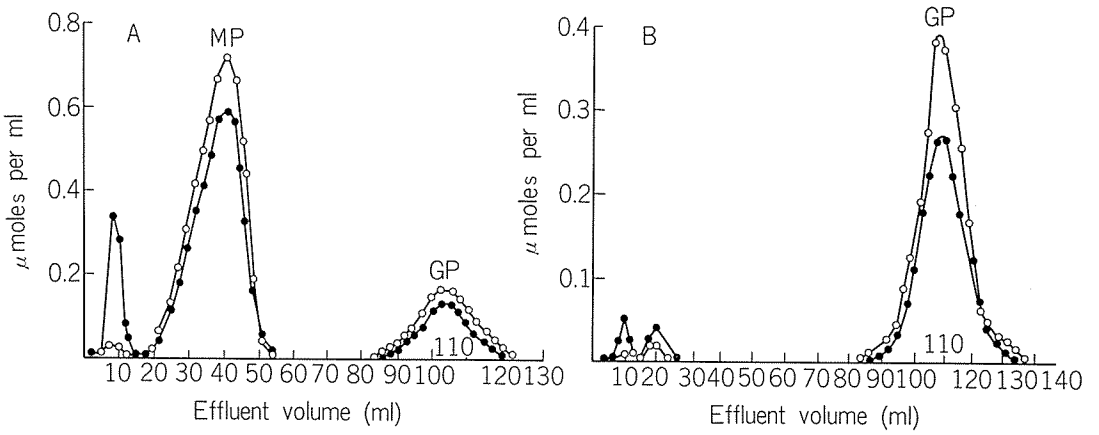

FIGURE 2. Rechromatography of Fractions II and III on a Dowex-50×8 column (A and B, respectively). The column was eluted with deionized water. Column size: 1×32 cm. Total hexosamines (○——○), total phosphates (●——●).

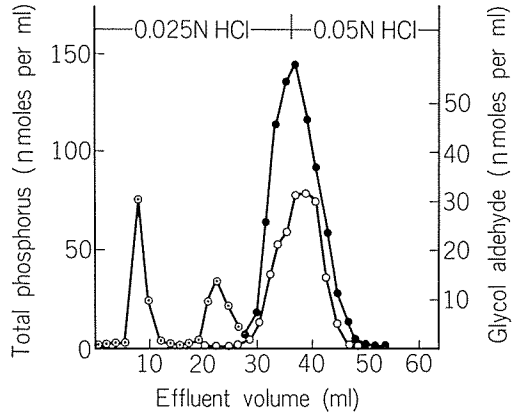

FIGURE 3. Chromatography of the GP fraction oxidized with $NaIO_4$ on a Dowex-1×8 column. The column was eluted with 35 ml volumes of 0.025 N and 0.05 N HCl. Column size: 1×15 cm. Glycolaldehyde (○——○), organic phosphate (●——●), inorganic phosphate (⊙——⊙).

with minor modifications. The GP fraction (3 μmole equivalents as total hexosamine) was oxidized with 10 μmoles of sodium metaperiodate in 1.1 ml of reaction mixture for 48 hr at room temperature, in the dark, and then the sample was applied to a Dowex-1×8 (Cl^- form, 200–400 mesh) column and the column was eluted successively with 35 ml volumes of 0.025 N and 0.05 N HCl. Although the molar ratio of organic phosphate to glycolaldehyde (Dische and Borenfreund, 1949) was far from unity, these compounds were eluted as a single peak with 0.05 N HCl (Fig. 3). The elution profile coincided with that of the oxidation products of authentic glucosamine 6-phosphate.

Hydrolyzates of the cell walls of various gram-positive bacteria (in 6 N HCl at 100 C, for 14 hr) were applied to the column of the amino acid autoanalyzer and eluted with the pH 3.25 buffer. Their contents of glucosamine 6-phosphate and muramic acid 6-phosphate were estimated by measuring the peak areas of materials with retention times of 22 and 18 min, respectively, by the ninhydrin reaction. The molar concentrations of these hexosamine 6-phosphates were calculated on the basis of the molecular extinction coefficients of authentic specimens. Appropriate corrections were made for destruction of materials during acid hydrolysis (2.53 and 2.67 for glucosamine 6-phosphate and muramic acid 6-phosphate, respectively) calculated from the half lives of the GP fraction (for glucosamine 6-phosphate) and the MP fraction (for mura-

TABLE 1. *Contents of glucosamine 6-phosphate and muramic acid 6-phosphate in cell walls of various gram-positive bacteria*[a]

Bacterial species (strain)	Glucosamine 6-phosphate[c]	Muramic acid 6-phosphate[c]	Muramic acid[c]	Glucosamine[c]	Glutamic acid
Gaffkya tetragena (ATCC 15292)	0.07	0.10	0.68	0.58	1.00 (688)[b]
Sarcina lutea (IFO 3232)	0.10	0.05	1.18	1.17	1.00 (363)
M. lysodeikticus (NCTC 2665)	0.02	0.02	1.11	0.79	1.00 (818)
Staphylococcus aureus (Copenhagen)	0.10	0.08	0.92	1.13	1.00 (439)
Streptococcus mutans (FA-1)	0.43	0.29	1.05	0.68	1.00 (223)
S. mutans (SS-9)	0.48	0.34	0.88	0.65	1.00 (227)
S. mutans (GS-5)	0.17	0.16	0.83	0.59	1.00 (278)
S. mutans (OMZ 65)	0.10	0.10	1.13	0.70	1.00 (339)

[a] The data are expressed as moles per mole of glutamic acid.

[b] The values in parentheses are nmoles of total glutamic acid residues per mg cell wall.

[c] Factors of 2.53, 2.67, 2.15 and 1.29 were used to correct for destruction of glucosamine 6-phosphate, muramic acid 6-phosphate, muramic acid and glucosamine, respectively, during acid hydrolysis (correction factors for muramic acid and glucosamine were taken from Kotani and Shimono, 1976).

The bacterial strains of which the contents of glucosamine 6-phosphate in the cell walls were zero or less than 0.01 mole per mole of glutamic acid residue were as follows: *Bacillus megaterium* (KM), BCG (Takeo), *C. diphtheriae* (PW 8), *Lactobacillus plantarum* (ATCC 8014), *S. mutans* (OMZ 176, 6715, HS-1, E-49, BHT, C67-1, C67-25, Ingbritt, JC-1 and JC-2), *Streptococcus mitis* (CHT), *Streptococcus sanguis* (10556 and 10557), *Streptococcus salivarius* (JC-6 and NCTC 8618), *Streptococcus faecalis* (NCTC 775), *Streptococcus faecium* (NCTC 7171). (Streptococcal cell wall specimens were kindly supplied by Dr. S. Hamada and his colleagues of this Department).

mic acid 6-phosphate) of *M. lysodeikticus* cell walls, measured by hydrolyzing the fractions for various times in 6 N HCl at 100 C. Table 1 shows the molar ratios of glucosamine 6-phosphate to glutamic acid residues in the cell walls, with those of muramic acid 6-phosphate, muramic acid and glucosamine. These results show that the cell walls of gram-positive cocci contained a significant amount of glucosamine 6-phosphate besides muramic acid 6-phosphate, whereas the walls of gram-positive bacilli contained a comparable amount of muramic acid 6-phosphate to that in coccal cell walls, but no appreciable glucosamine 6-phosphate.

The function of the glucosamine 6-phosphate in the cell walls is unknown. In a previous study (Iwata et al., 1977) we found that when the cell walls of *M. lysodeikticus* were treated with the endo-*N*-acetylglucosaminidase and then subjected to chromatography, some fractions contained only glucosamine 6-phosphate and other fractions contained both glucosamine 6-phosphate and muramic acid 6-phosphate. This finding suggests that glucosamine 6-phosphate may be involved in the linkage of a glycan portion of *M. lysodeikticus* peptidoglycan to special structures in the cell wall, such as muramic acid 6-phosphate. While this study was in progress, the identification of glucosamine 6-phosphate as a component of *M. lysodeikticus* cell walls by periodate oxidation and methylation studies was reported by Nasir-ud-Din, et al. (1977). It may be added here that direct linkage of arabinogalactan, a special structure of *Mycobacterium*, to the *N*-acetylglucosamine residue in the peptidoglycan of BCG cell walls was suggested by Kanetsuna and San Blas (1970) on the basis of the finding that

part of the arabinogalactan-peptidoglycan complex contained no muramic acid residues. However, they did not show that a glucosamine phosphate participated in the linkage.

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